Monitoring the degree of Frozen Denaturation of Bovine Myosin & its Subfragments by Competitive indirect Enzy Linked Immunosorbent Assay (Ci-ELISA)

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ABSTRACT : *To monitoring frozen denaturation of bovine myosin, we purified myosin whole molecule (MWM) and subfragments, heavy meromyosin (HMM) S-1 (S-1 ; 90 kDa) and light meromyosin (LMM, S-3 ; 69 kDa), restrictedly proteol by α-chymotrypsin. Rabbits were immunized with S-1, S-3 and MWM and theit IgGs were purified. We obtained good Ci-EL condition for quantifying these antigens, when all IgGs were diluted 1:5,000 (0.2 ug/ml), tracer was diluted 1:10 (l.s.d.;p<0.01). We studied the frozen denaturation of bovine myosin under two different conditions ; Exp-1) storage during fr periods, Exp-2) repeated thawing refreezing treatment. this study showed that the amount of MWM in Exp-1 were decreased 31.6, 31.4, 49.4 and 34.2% at 6 months, while after 6 time thawing that in Exp-2 were to 14.8, 9.6, 14.6 and 12.6% at -10, -20, -50 and -80℃, respectively (l.s.d.;p<0.05). The immunoreactivities of anti-S-1 IgG with MWM from Exp-1 and Exp-2 w not easy to monitor clearly. The reactivities of anti-S-3 IgG were very similar to those of MWM. In Exp-1, the reactiv decreased to 37.7, 32.2, 52.5 and 29.0% and that of Exp-2 was to 11.8, 15.6, 32.2 and 23.7% at -10, -20,-50 and -8 respectively (l.s.d.;p<0.05).*

INTRODUCTION : During frozen storage, meat have suffered decrease of protein solubility, denaturation of myofibrillar prote (mainly myosin), undisirable change of meat colour, and hardness of texture by loss of meat juice, these factors injure quality meat[1-5], some reports have presented that frozen treatment and the changes during frozen periods were affected to qualities animal and fish meat[6,7], denaturation of muscle proteins, especially myofibrils served important parts of the decline of quality[2]. Many researchers have proposed that the most demaged sites in muscle cells by frozen are myofilaments and injure myosin molecule is so large by ice crystalization and recrystalization occured by refreezing during frozen processing[8,9]. Theref frozen meat is sailed cheaper than chilled meat. But frozen meat have been thawed, distributed and sold as fresh chilled m irregally by retail trades in korean markets. Immunological method will be able to differentiate denatured proteins from nativ We assumed denatured parts by frozen seem to be scattered about MWM or any certain parts rather than one site in the view structural property of myosin. In these studies, we produced polyclonal antibodies immunoresponded to bovine skeletal mu MWM, S-1 and S-3, separated and purified IgG, and established Ci-ELISA. Using this assay, we investigated degree denaturation of bovine myosin occured by frozen for differentiation of frozen-thawed beef and fresh beef.

MATERIALS & METHODS : Myosin were purified from bovine M.*Semitendinosus* modified by Choi's[11] method. Purity checked by SDS-PAGE (10% homogenous and 5-15% gradient acrylamide) in Claeys *et al*[12], staining method. Modified by Wee and Pope[13], we treated α-chymotrypsin for producing myosin subfragments, S-1 and LMM. Purified MWM, S-1, and S-3 w used to produce antiserum and to establish Ci-ELISA. Rabbits were immunized by intradermal injection of purified immunog emulsified with an equal volume of adjuvant as 100 ug/ml of final concentration. Each blood was collected by heart-punc antisera was separated. Antiserum were afinity-chromatographed on a Protein A High Trap column (5ml) to separate IgG specific to antigen. By Lee *et al*[14]'s assay method, Ci-ELISA was established. Standard curves for quantification of Ags were obtain under fixed analytical condition. M.*Semitendinosus* of domesticated korean cattle was used in this investigation. For Experiment (Exp-1), whole muscles were cut to 8-10 cm diameter and 1 cm thickness, about 60 grams; for Experiment 2 (Exp-2), whi muscle were cut to 8-10 cm diameter and 15 cm thickness, about 900 grams, all samples were correctly weighe vacume-packaged, frozen at -10 ± 1℃ , -20 ± 1℃, -50 ± 1℃ and -80 ± 1℃ deep freezer as method of cold-air circulati and stored until request for experiments. All samples were prepared to triplicates, experiments were performed at 1 month inter Solubility of myofibrillar proteins (MPS) was modified by methods of Petrovic *et al*[15], measured by BCA using differently dilut BSA solution as standard protein concentration. Filtrates were used at MPS and ELISA test. Sample solutions for detecting Ag were diluted to 10 times with 0.6 M NaCl, 0.01 M PB, pH 7.4, 50 ul of diluents was added to 3 wells of plates coated a blocked. We evaluated data by the method of limited standard deviation (l.s.d) in SAS program.

RESULTS & DISCUSSION : By Choi[11] 's method and Weeds and Pope[13] 's method, MWM, S-1, and S-3 were obtained. T result of purity of each Ag was higher than 90% by SDS-PAGE (Data not shown). In the condition of fixed concentrations coated myosin (10 ug/ml), primary IgG (1:5,000), and secondary IgG (1:20,000), all Ags could be detected in the range of ug/ml to 125 ug/ml (l.s.d.;p<0.01), limit of detection was 0.1 ug/ml (l.s.d.;p<0.01) (fig. 1). Opportunity of reaction of IgG w Ag of 10 ug/ml in plates are varied by concentration of added standard Ag. When various concentration of Ag and coated (10 ug/ml) and primary IgG (0.2 ug/ml) were prepared, at 490nm spectrometric value (B_1) obtained from reaction with stand Ag could be compared with control, not added Ag, (B_0), and the values were appeared as percentage, $B_1/B_0 \times 100$, and obtain values could be expressed as standard curves by semi-log. Denaturation of myofibrillar proteins occurred by frozen had be investigated by DSC and MPS[1, 2, 15]. In our research of Exp-1, reactivity of anti-MWM IgG and MWM from samples stored -10, -20, -50 and -80℃ were decreased to 31.67, 31.42, 49.46 and 34.27% after 6 mon storage, respectively (l.s.d.;p<0.05; F 2-Ⅰ-A). In comparison with the change of MPS, two results were not appeared with any relationship during 6 months storag Exp-2 were very severely changed from control (Fig. 2-Ⅰ-B). At only 2 times thawing, samples stored at -10℃ and -20℃ w appeared to decrease of concentration of MWM of about 50% compared to control, but MPS was about 91% above (Table All samples except for that at -50℃ were presented from 33.21% (at -20℃) to 43.6% (at -10℃) at 3 times thawing. All sampl at 6 times thawing, Abs were immunoreacted with their epitopes to 14.8, 9.6, 14.6 and 12.6% below at -10, -20, -50 and -80 respectively (l.s.d.;p<0.05). Rate of storage temperature of -20℃ was the most severely affected and denatured by both treatme while that of -50℃ the least. There was not any relationship with solubility in comparison of change of protein concentration. Exp-1, reactivity of anti-S-1 IgG and S-1 stored at -10℃ and -50℃ was disappeared any differences with control (Fig. 2-Ⅱ-A Results of Exp-2 were very differed from Exp-1 (Fig. 2-Ⅱ-B). All treatments were decreased to 3 times thawing, even thou

not same. However, at 6 times thawing detected amounts of S-1 were not changes compared with control. The change of S-3 was apparently shown (Fig. 2-Ⅲ-A, -B). The degree of immunoreaction of Exp-1 was reduced to 37.73, 32.28, 52.54 and 29.02% and that of Exp-2 was to 11.89, 15.60, 32.24 and 23.76% for 6 month storage at -10, -20, -50 and -80℃, respectively (l.s.d.;$p<0.05$). Change at Exp-1 occurred at all treatments was similar with tendency, the result of MWM (Fig. 2-Ⅰ-A, -Ⅲ-A). Decrease of reactivity of anti-S-3 IgG and S-3 simultaniously started at frozen storage, samples at -20℃ were the most severely demage. The decrease of immunoresponse of S-3 at Exp-2 was same tendency with Exp-1, but more viorent (Fig. 2-Ⅲ-B). All treatments except for samples at -50℃ were decreased to 35% above at only 2 times thawing, especially 50% at -20℃ (l.s.d.;$p<0.05$). Denaturations of S-3 through Exp-2 were occured more, when stored higher temperatures. Samples at -10℃ and -20℃ were denatured to more about 10 - 15% than samples of lower temperature. These results showed that denaturation of myosin were mainly occurred at the part of myosin rod during frozen storage. We could compare our results with Wagner and Anon[2], because peak Ⅰ and Ⅱ of DSC thermogram were presented as thick filament of myofibrillar proteins. Report by DSC supported our results, decrease of peak Ⅱ was estimated as denaturation of LMM[16]. That ice crystal is formed in cetain positions of muscle cell and affect fibres may be affected by storage temperature and frozen and thawing methods. Morphology of myosin rod differs from that of myosin head and coiled coil helical structure may be able to be injured by frozen more easily.

CONCLUSION : Myosin molecules were destructured and structurally denatured by frozen treatment, when monitoring by Ci-ELISA established by us. Myosin in meat at storage temperature of eutectic point (-40 - -50℃) were denaturated smaller than that of other temperatures, beef slices stored at -20℃ were the most severely demaged. Frozen denaturation of myosin subfragments produced by α-chymotryptic proteolysis were more severely demaged in LMM and results to S-1 could be not surely determined by Ci-ELISA.

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TABLE & FIGURES

Table. 1. Change of MPS of beef cuts in the various zones of storage temperature at Exp-1 and Exp-2

(mg/ml, %)

Storage (mon)	Storage temp (℃)	0	1	2	3	4	5	6
Exp-1	-10	2.86 100*	3.28 114.69	3.13 109.44	2.81 98.25	2.65 92.66	2.55 89.16	2.54 88.81
	-20	3.17 100*	3.46 109.15	3.64 114.83	3.58 112.93	3.50 111.46	3.26 102.84	2.89 91.17
	-50	3.09 100*	3.47 112.30	3.69 119.42	3.65 118.12	3.54 114.56	3.29 106.47	3.14 101.62
	-80	3.14 100*	3.39 107.96	3.71 118.15	3.47 110.51	3.50 111.46	3.43 109.23	2.81 89.49
Exp-2	-10	2.93 100*	2.68 91.47	2.66 90.78	2.60 88.73	2.36 80.55	1.93 65.87	1.99 67.92
	-20	3.07 100*	2.94 95.77	2.97 96.74	2.92 95.11	2.99 97.39	2.59 84.36	2.53 82.41
	-50	2.42 100*	2.59 107.02	2.65 109.50	2.61 107.85	2.51 103.72	2.49 102.89	2.32 95.87
	-80	2.99 100*	3.19 106.69	3.05 102.01	3.01 100.69	2.95 98.66	2.76 92.31	2.65 88.63

(l.s.d.;$p<0.05$)

a : these values were calculated in the percentage of detected results of treatments to control (fresh beef).

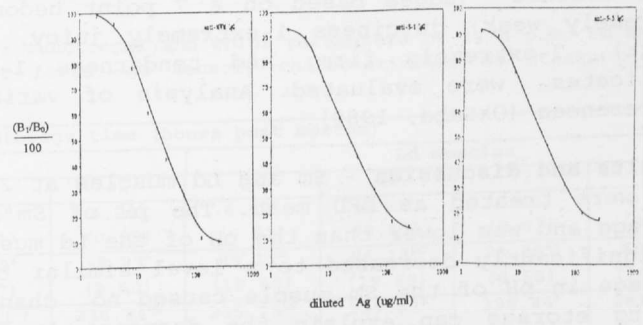

Fig. 1. Standard curves for quantifying MWM, S-1 and S-3, and determinating denaturation of myosin molecules by freezing treatments (l.s.d.; $p<0.01$)

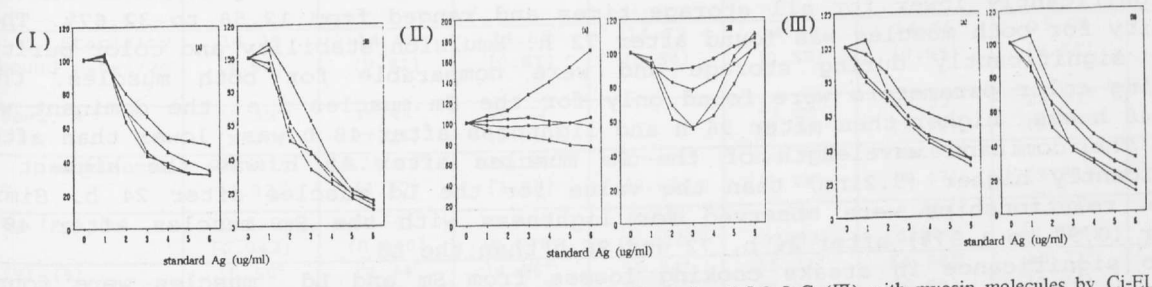

Fig. 2. The changes of immunoresponse of anti-MWM IgG (Ⅰ), anti-S-1 IgG (Ⅱ) and anti-S-3 IgG (Ⅲ) with myosin molecules by Ci-ELISA. (A) Exp-1, (B) Exp-2. M_1 (treatments) indicates detected concentration of myosin extracted from frozen sample, and M_0 (control) indicates concentration of myosin extracted from fresh beef. 10 samples were investicated, triplicates were determinated (l. s. d.; $p<0.05$) (■) at -10℃, (□) at -20℃, (×) at -50℃, and (▲) at -80℃].